DETERMINATION OF THE EFFECTS AND MECHANISMS OF ACTION OF COMPOUNDS RELATED TO VARIOUS MOLECULAR PATHWAYS ON THE REGENERATION OF β-CELLS *IN-VIVO*

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DETERMINATION OF THE EFFECTS AND MECHANISMS OF ACTION OF COMPOUNDS RELATED TO VARIOUS MOLECULAR PATHWAYS ON THE REGENERATION OF β-CELLS *IN-VIVO*

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LIST OF SYMBOLS AND ABBREVIATIONS

dpf	Days post-fertilization
hpf	Hours post-fertilization
DMSO	Dimethyl sulfoxide
GFP	Green fluorescent protein
CFP	Cyan fluorescent protein
RFP	Red fluorescent protein
DSR	DsRed
Tg	Transgenic
NTR	Nitroreductase
ins:Kaede	*insulin:kaede* transgenic
MTZ	Metronidazole
PTU	Phenylthiourea
EdU	5-ethynyl-2'-deoxyuridine
DMSO	Dimethyl sulfoxide
TBK-1	TANK-binding kinase 1
IKKε	Inhibitor of nuclear factor kappa-B kinase subunit epsilon
PEM	PIPES EDTA

ABSTRACT

Chemicals associated with anti-inflammatory biological signaling pathways are known to be effective methods of alleviating the symptoms for numerous diseases and medical conditions, including diabetes. Recently, an increasing number of studies are analyzing biological signaling compounds in the context of having the potential to directly stimulate regeneration of pancreatic β-cells. Few efforts have been successful in demonstrating and characterizing the relationship between compounds associated with anti-inflammatory pathways and the proliferation and regeneration capability of β-cells. This study aimed to test the effects of chemicals with anti-inflammatory effects on the up-regulation of insulin regeneration in zebrafish embryos. The ultimate goal was to discover specific anti-inflammatory drugs that have the potential to treat diabetes by directly increasing the size and insulin-secreting functionality of the β-cell mass, as well as to determine their mechanisms of action. It was discovered that the small molecule BX-795, which acts as an anti-inflammatory agent and inhibits the catalytic activity of the protein kinases tank-binding kinase 1 (TBK1) and Inhibitor of nuclear factor kappa-B kinase subunit epsilon (IKKε) of the toll-like receptor biological signaling pathway, has great potential as a successful inducer of β-cell regeneration following ablation of β-cells. This compound and the toll-like receptor pathway have potential to be involved in the reversal of Type I diabetes.

CHAPTER 1

INTRODUCTION

Diabetes is an endocrine disease characterized by abnormally high blood glucose levels. It is the seventh leading cause of mortalities and increases chance of death by 50% in adults in the United States. This statistic does not include deaths caused by complications triggered by diabetes, such as heart disease (which is the leading cause of death in the United States), eye complications, kidney disease, and neuropathy[1]. Type I diabetes is a genetically inherited autoimmune disease that causes destruction of insulin-released β-cells in the pancreas, leading to insulin deficiency and high blood glucose levels[2]. Type II diabetes is caused by insulin-resistance, which involves impaired detection of the normal levels of insulin being produced by β-cells and therefore an increased demand for insulin via compensatory β-cell function[3].

Currently, commonly diabetes-specific treatments include insulin supplements and the oral drugs metformin, pioglitazone, and sitagliptin. Each of these treatments has a unique mechanism of action associated with biological signaling pathways, yet none has its most potent effect on targeting the underlying cause of diabetes by increasing β-cell function[4 5 6]. A novel idea for treatment of diabetes, particularly Type I diabetes, is reversing its underlying cause via stimulation of β-cell proliferation and regeneration. Studies on the proliferation and differentiation of β-cells that utilize model organisms *in vivo* show great potential for reversing the outcome of β-cell death in humans. The zebrafish *Danio rerio* is an effective model organism for such studies because it is a vertebrate that has externally developing embryos, it can be mutated easily, and it has rapid embryonic development (**Figure 1**). Additionally, the optical clarity of zebrafish embryos allows for visualization of the organism's vital organs through its developmental stages utilizing fluorescent protein tagging[7]. The structure and function of the

zebrafish hepatopancreatic system closely resemble those of humans; therefore, regenerative responses of the zebrafish to chemical and physical stimuli show great potential in studies of diabetes treatment in humans[8].

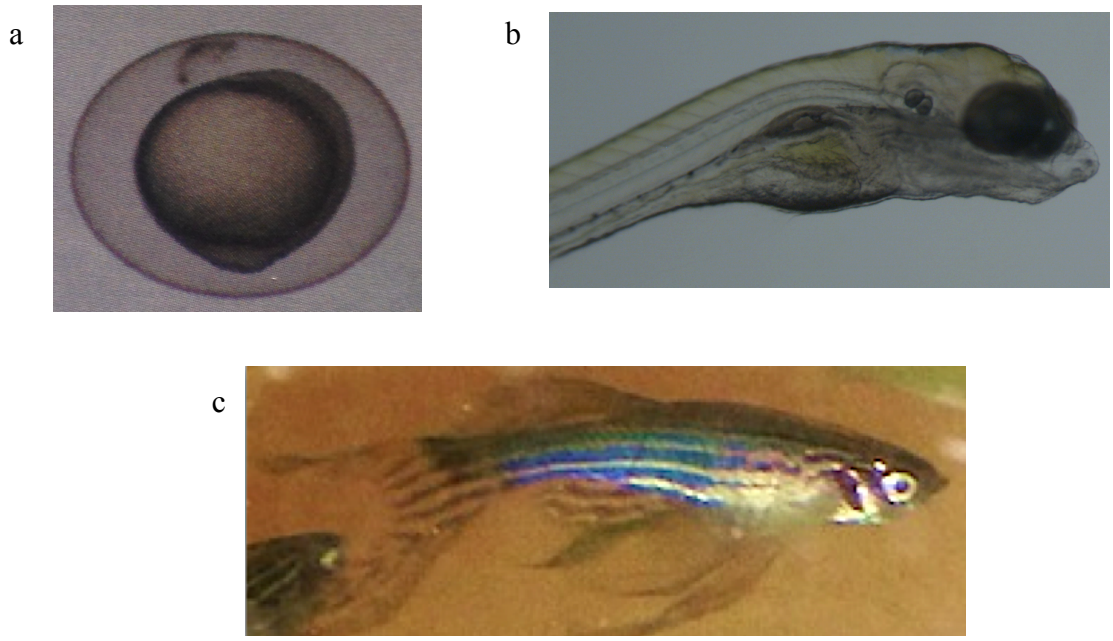


Figure 1: Images of zebrafish. a) Zebrafish embryo in the first 24 hpf; b) Zebrafish embryo at 6 dpf; c) Adult zebrafish.

It is known that drugs that exert anti-inflammatory effects on biological signaling pathways have the potential to reverse the incidence and delay the onset of Type II diabetes. However, the specific relationship between anti-inflammation and the regenerative properties of β-cells is yet to be established. This study aims to test the effect of chemicals with anti-inflammatory effects on the up-regulation of insulin regeneration in zebrafish embryos. The ultimate aim is to identify anti-inflammatory drugs that have the potential to treat diabetes by directly increasing the size and insulin-secreting functionality of the β-cell mass.

CHAPTER 2

REVIEW OF LITERATURE

Chemicals associated with anti-inflammatory biological signaling pathways are known to be effective methods of alleviating the symptoms for numerous diseases and medical conditions, including diabetes. Recently, an increasing number of studies are analyzing biological signaling compounds in the context of having the potential to directly stimulate regeneration of pancreatic β-cells. So far few efforts have been successful in demonstrating and characterizing the relationship between compounds associated with anti-inflammatory pathways and the proliferation and regeneration capability of β-cells[9].

Diabetes involves progressive β-cell death, which prevents the production of adequate levels of insulin to control serum glucose levels. An expanding body of evidence suggests that inflammation plays a significant role in the onset and progression of the disease. Many anti-inflammatory drugs with their most pronounced effects on non-diabetes ailments, including angiotensin-converting-enzyme inhibitors, metformin, aspirin, and thiazolidinediones, are known to the reduce the risk and alleviate the symptoms of Type II diabetes[10]. However, each of these drugs has a distinct mechanism of action that is not necessarily specific to targeting pancreatic β-cells.

Among the most promising anti-inflammatory compounds tested for effects on diabetes reversal and β-cell regeneration is the inhibitory neurotransmitter gamma amino-butyric acid (GABA). Numerous studies have shown that GABA also exerts significant function in the immune system and pancreatic islets of Langerhans, and it particularly acts on T-cells and macrophages to produce its anti-inflammatory effects[11]. Furthermore, GABA has been demonstrated to promote proliferation of human β-cells *in vitro*[12], increase DNA synthesis and

insulin release in the pancreatic cell line INS-1[13], regulate insulin secretion in response to fluctuating glucose levels, and reduce apoptosis in cultured cells[14]. GABA has been found to inhibit the activation of the transcription complex NF-kB in islet cells and more specifically to increase the expression of the SIRT1 gene to reduce apoptosis, a novel pathway of research for diabetes therapy[15].

Furthermore, a recent *in vivo* study found that compounds associated with the adenosine signaling pathway promote regeneration of β-cells[16]. In this study, over 5000 different chemical compounds associated with different biological signaling pathways were tested for their effects on β-cells in zebrafish embryos that were double positive for *Tg(ins:CFP-NTR)* and *Tg(ins:Kaede)*. Compounds that are both agonists and antagonists of adenosine signaling were found to increase the size of the β-cell mass following ablation, the most potent of these being the adenosine agonist 5'-N-ethylcarbox-amidoadenosine (NECA)[16]. These chemicals were confirmed to increase regeneration rather than proliferation of surviving β-cells by photoconversion of *Tg(ins:Kaede)*, as well proven to be successful in suppressing free glucose levels. NECA also had a modest effect on β-cell formation during normal development, without ablation[8]. Adenosine is an anti-inflammatory agent known to regulate tissue damage and repair[17]; therefore, it may impact the proliferation that results from the regenerative response to β-cell death. Furthermore, there is a relationship between the A2b gene locus and elevation of pro-inflammatory mediators that contribute to increased insulin resistance[18]. The study concluded that the anti-inflammatory pathways associated with adenosine signaling impact β-cell mass size and functionality, and stated that adenosine signaling may have several different mechanisms by which it can promote regeneration of β-cells[8].

It is known that proliferation of β-cells is induced by related classes of nucleotides and

nucleosides, including adenosine triphosphate (ATP) and adenosine, binding to receptor loci such as A2a and A2b. This sequentially causes an increase in cyclic AMP (cAMP) levels, activates protein kinase A, phosphorylates cAMP response element-binding protein, and binds to cAMP response elements in promoter regions of target genes to directly regulate transcription[19]. Although it is known that the binding receptors involved with proliferation are associated with inflammatory markers, a precise mechanistic relationship between anti-inflammation and β-cell proliferation is yet to be discovered.

The drugs metformin, pioglitazone, and sitagliptin are currently common treatments for Type II diabetes. Each of these medications has a distinct mechanism of action, yet none have their most potent effect on increasing β-cell function[20 21 22]. Nonetheless, studies have shown that each of them has an up-regulatory effect on markers of β-cell function in patients with Type II diabetes[23] and that each has an anti-inflammatory effect from their mechanisms[24 25 26]. Furthermore, other commonly prescribed anti-inflammatory drugs, particularly statins for hypercholesterolemia and ACE inhibitors for hypertension, also have anti-diabetic properties[27]. Few findings exist that analyze the effects of these drugs on β-cell mass size and hence their applicability in directly treating Type I diabetes.

One of the most useful and high-throughput methods of studying size and function of β-cells is by utilizing transgenic zebrafish. One powerful system to utilize is the double transgenic *Tg(ins:CFP-NTR);Tg(ins:Kaede)*. *Tg(ins:CFP-NTR)* contains a fusion protein of cyan fluorescent protein (CFP) and the enzyme nitroreductase under the insulin promoter. Nitroreductase (NTR) is an enzyme derived from *E. coli* that converts the compound metronidazole (MTZ) to a toxic product, which results in apoptosis of insulin producing β-cells. The ablated cells, upon removal of MTZ, have the ability to recover from ablation.

Tg(ins:Kaede)) expresses the Kaede protein, which originates from stony coral and is characterized by very bright green fluorescence. Kaede also has the property of being photoconvertible. Upon ultraviolet irradiation, the green color of the protein can be converted to red (**Figure 2**).

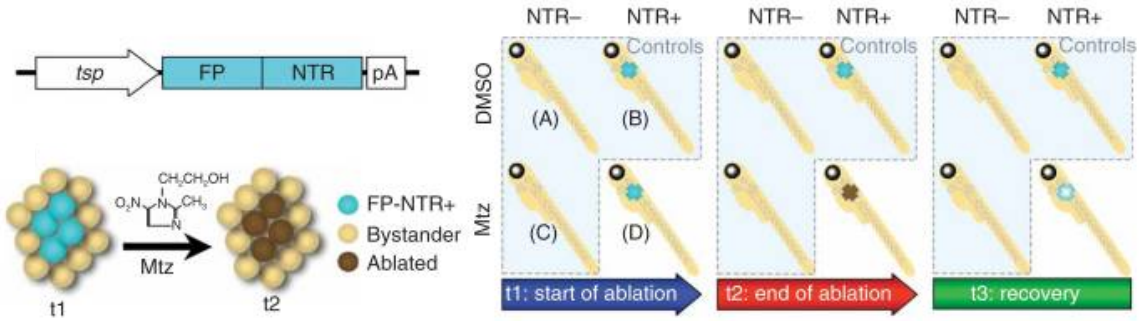


Figure 2: Experimental design of tissue-specific ablation using the CFP-NTR transgenic.[28]

In a preliminary study, the 73 compounds of the Selleck stem cell library were tested for their effects on the regeneration capability of the β-cell mass in zebrafish embryos. This was assessed utilizing the *Tg(ins:CFP-NTR);Tg(ins:Kaede)* system with MTZ treatment. The nitroreductase protein labeling of the β-cells allows for ablation, or death, of the β-cell mass upon treatment with the compound metronidazole (MTZ). It was discovered that the small molecule BX-795, which acts as an anti-inflammatory agent and inhibits the catalytic activity of the protein kinases tank-binding kinase 1 (TBK1) and Inhibitor of nuclear factor kappa-B kinase subunit epsilon (IKKε) of the toll-like receptor signaling pathway, has been found to have an upregulatory effect on the regeneration of β-cells in the zebrafish model organism. The drug amlexanox, an asthma drug that is a TBK1 and IKKε inhibitor, has been found to alleviate obesity and obesity-induced health problems in mice and is currently being investigated[29]. So far few other studies have honed in on the effect of this signaling pathway on diabetes causation and

treatment. None have analyzed the effect of inhibition of TBK1 and IKKε on the functionality of β-cells.

With the wealth of knowledge that exists regarding the effect of inflammation on diabetes, it is clear that a significant relationship exists between inflammation and β-cell viability and functionality. A step forward toward a clearer understanding of this relationship is the discovery of a new biological mechanism that relates these two.

The proposed study further addresses the necessity and potential for diabetes treatments that involve stimulating the health and action of β-cells by examining the effect of anti-inflammatory compounds on the proliferation and regeneration capacity of β-cells *in vivo* using zebrafish embryos. It aims to test the effect of a variety of compounds, particularly those that inhibit the function of TBK1 and IKKε, on the regeneration capacity of ablated β-cells and the normal development of β-cells.

If the compounds tested prove to be consistently successful in increasing the size and function of the β-cell mass in zebrafish, the discovery could pave the way for new studies and therapies that target these parts of the toll-like signaling pathway to counter the underlying cause of diabetes. Furthermore, this study aims to test the effect of many commonly used anti-inflammatory diabetes drugs on β-cell regeneration, to find potential links between the mechanism of BX-795 and those of these compounds.

CHAPTER 3

EXPERIMENTAL PROCEDURES

GENERAL CHEMICAL SCREENING PROTOCOL

In the majority of the experiments in this study, the nitroreductase-metronidazole (NTR-MTZ) system was applied to the double transgenic *Tg(ins:CFP-NTR);Tg(ins:Kaede)*. An overview of the full process is displayed in **Figure 3**.

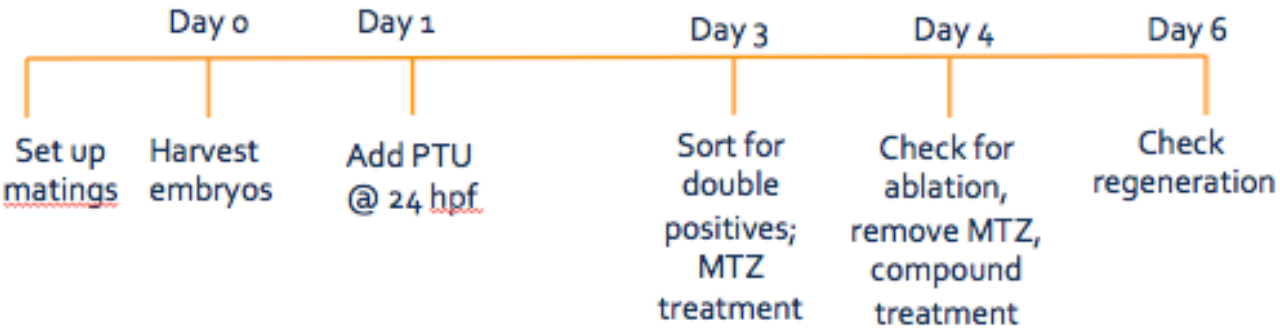


Figure 3: Summary of general timeline for chemical screening.

Reagents Egg water was prepared by combining double-deionized H_2O, calcium sulfate obtained from Acros Organics, Instant Ocean, and methylene blue. Phenylthiourea (PTU) was obtained from Sigma Aldrich. The initial set of 73 compounds tested was the Stem Cell Library obtained from Selleck Chemicals. Dimethyl sulfoxide (DMSO) was obtained from Fisher Scientific (BP231-1). The metronidazole (MTZ) was obtained from Sigma Life Science (M1547-25G).

Mating of zebrafish to yield transgenic embryos The procedure for chemical screening followed a 7-day timeline (Figure 3). On average, each week, between ten and twelve matings were set up between zebrafish that were double-positive for *Tg(ins:CFP-NTR);Tg(ins:Kaede)* by

placing one female with two males or two females with one male in a mating tank to increase chances of productivity of the cross. The next morning, the tanks were checked for presence of eggs. On average, about half the crosses set up for each round were productive; however, the fertility of the fish was highly dependent on their age, diet, and health condition. The eggs were harvested, cleaned, sorted into petri dishes with 80 eggs and approximately 20 mL of egg water each, and incubated at 35°C for the next 24 hours.

Prevention of pigmentation At 1 day post fertilization (dpf), the petri dishes were each cleaned, by replacing the egg water and removing dead embryos. A 20X solution of PTU (1 mL) was added to each of the plates to prevent pigmentation buildup in the embryos, and the plates were incubated at 35°C for the next 48 hours.

Sorting of double positive embryos and MTZ ablation At 3 dpf, the embryos were viewed under the Leica M205 fluorescence microscope under both the GFP and CFP channels to separate embryos that were double positive (the CFP and GFP were both visible, **Figure 4**). To ablate the β-cells, MTZ solution (15 mM) was prepared by combining 20 mL of egg water, 20 µL DMSO, 1 mL 20X PTU, and 0.1 g of solid MTZ per petri dish containing approxomiately 60 embryos. The bottle in which the solution was prepared was wrapped in aluminum foil to prevent light-sensitive MTZ from losing its activity. The MTZ solution was dispensed into the petri dishes and the petri dishes were wrapped in aluminum foil and incubated at 35°C for the next 24 hours.

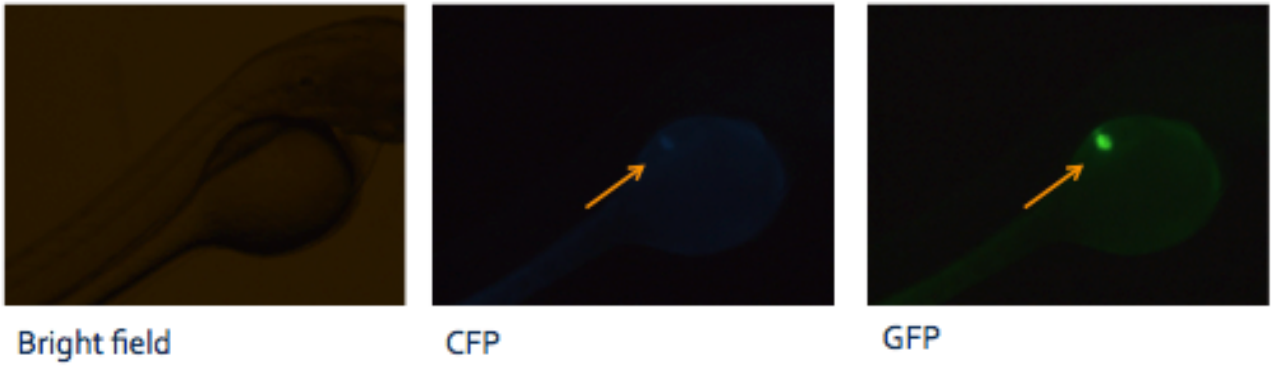


Figure 4: Images of double positive embryo at 3 dpf prior to addition of MTZ. The embryo under the bright field, cyan fluorescent protein filter (*Tg(ins:CFP-NTR))*, and green fluorescent protein filter (*Tg(ins:Kaede))* are displayed. A bright dot representing β-cell mass is clearly visible under both fluorescent filters.

Confirmation of ablation and treatment of compounds At 4 dpf, the embryos were viewed under the GFP channel of the fluorescent microscope to sort for fully ablated embryos, which had no GFP visible (**Figure 5**). The MTZ was removed from the water of the embryos by washing the embryos 3 to 4 times with egg water. The embryos were sorted into 24-well plates containing approximately 6 embryos per well in 400 µL of 1X PTU. A chemical from the Selleck stem cell library dissolved in DMSO at a concentration of 25 µM was added to each well. Wells containing embryos exposed to DMSO served as the negative control and wells containing embryos exposed to LY411575, an inhibitor of the Notch signaling pathway, at a concentration of 12.5 µM served as the positive control.

Figure 5: Images of double positive embryo 4 dpf following MTZ-mediated ablation. The embryo under the bright field, cyan fluorescent protein filter (*Tg(ins:CFP-NTR))*, and green fluorescent protein filter (*Tg(ins:Kaede))* are displayed. A bright dot representing β-cell mass is no longer visible under either of the fluorescent filters.

Visualizing regeneration Forty-eight hours later, effects of the compounds on regeneration of β-cells was observed by comparing the relative size of the β-cell mass of the fish exposed to the test conditions to those exposed to both the negative control and the positive control. Chemicals that caused the embryos to show a significant increase in the size of the β-cell mass as visible under the fluorescence microscope were recorded. Photos were taken using a Leica DFC425 C digital camera and Leica software. Compounds that were questionable or hopeful for increased insulin regeneration were re-screened. An overview of this process is displayed in **Figure 3**.

SUPPLEMENTAL STEPS TO CHEMICAL SCREENING PROTOCOL

Dilution of chemical compounds The chemical compounds utilized in this study were obtained in powder form. Therefore, it was necessary to dilute them in DMSO to utilize them as treatments for the embryos. Each chemical compound stock solution was prepared at a concentration of 10 mM. The initial working concentration utilized for the treatments was 25 μM. The working concentrations were either increased or decreased depending on viability

results seen in experiments or based upon concentrations utilized in literature.

Table 1: List of compounds stock solutions prepared

Compound Name	Function	Source	Working concentration
BX-795	TBK1/ IKKε inhibitor	Selleck Chemicals	25 mM
Amlexanox	TBK1/ IKKε inhibitor	Sigma Aldrich	25 mM
CYT-387	TBK1/ IKKε inhibitor	Selleck Chemicals	25 mM
MRT67307	TBK1/ IKKε inhibitor	Calbiochem	25 mM
NECA	Adenosine A1 and A2 receptor	Tocris Biosciences	25 mM

Photoconversion of Tg(insulin:kaede) MTZ treatment (15 mM) does not always ablate all of the β-cells following a 24-hour treatment time, and treating the embryos for longer than 24 hours tends to decrease the viability of the embryos. To track β-cells that were not fully ablated following MTZ treatment, the GFP of the Kaede protein was converted from green to red prior to MTZ treatment by exposure to ultraviolet radiation. With photoconversion, all of the remaining cells following MTZ treatment are red, while those that regenerate are green. This allows for clearer distinguishing between surviving and regenerated cells, and therefore allows for determination of whether increased β-cell mass by certain drugs was a result of survival or regeneration. The compounds that were observed to increase regeneration of β-cells were re-tested following photoconversion of the Kaede protein marking the insulin cells of the zebrafish embryos. The general chemical screening procedure was followed through 3 dpf. Double-positive embryos were sedated by adding 3-aminobenzoic acid ethyl ester (tricaine) solution at pH 7 to the surrounding egg water. The embryos were placed into a 35 mm glass-bottom petri dish. Using the Zeiss LSM 700-405 confocal microscope, the focus was adjusted to β-cell mass with Kaede expression, particularly to the plane demonstrating the greatest area and brightest expression of the protein. The boundary of the β-cell mass was selected and a 405 nm UV light

was utilized for the bleaching of this region. The exposed cells transformed to a red color. If any yellow spots persisted, indicating the overlap of green and red cells, the bleaching experiment was performed a second time, but using fewer cycles to minimize damage to the embryo. The general chemical screening protocol was continued as written. Following MTZ treatment, compound treatment, and regeneration, the newly formed cells that originated from the cells that persisted following MTZ treatment were yellow in color under both the GFP and DSR filters (due to the overlap of green and red cells), while the regenerated cells that did not originate from the original cells were green in color under the both the GFP and DSR filters.

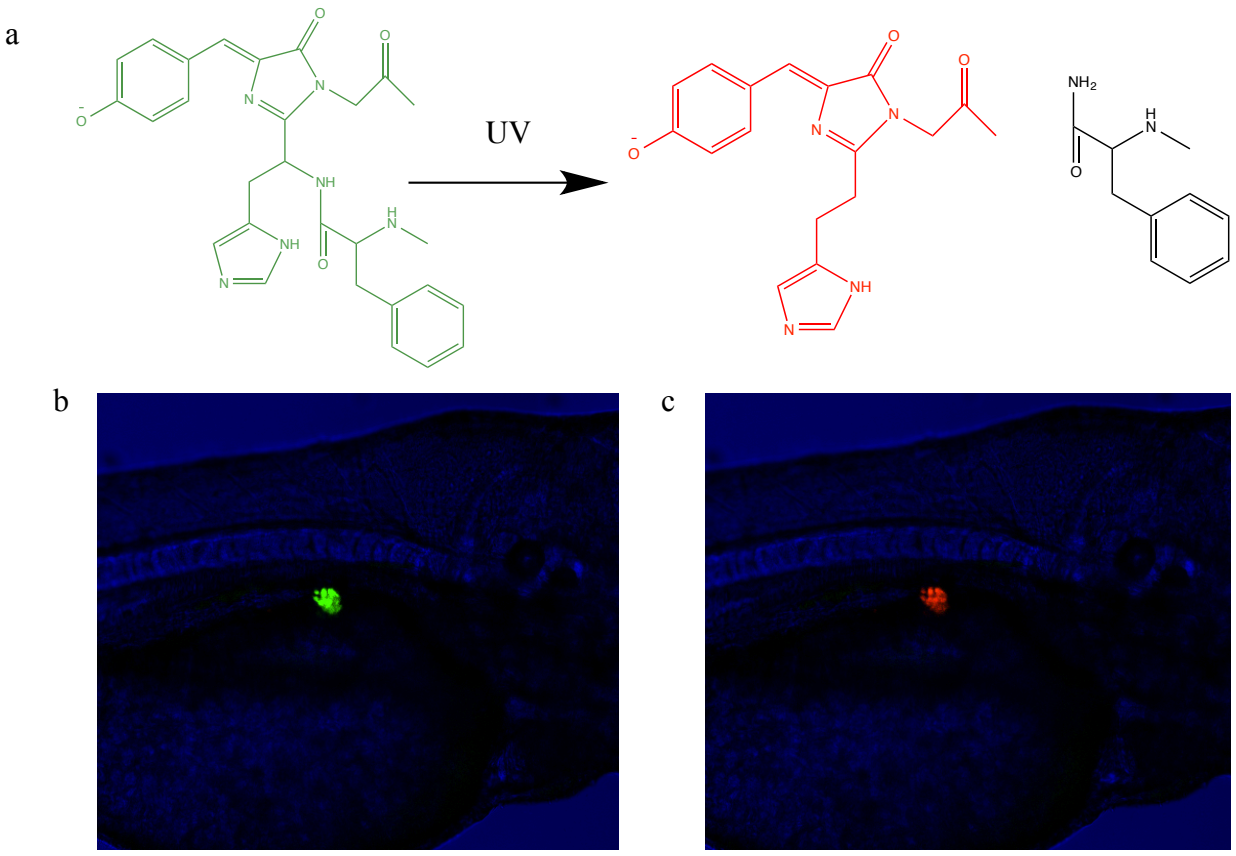


Figure 6: Photoconversion to Tg(ins:Kaede) embyros. a) Reaction that causes photoconversion. The reactant is a red chromophore, which is cleaved to form a red chromophore. b) Confocal image of embryo prior to photoconversion, showing GFP labeling of β-cells. c) Confocal image of embryo following photoconversion of GFP, showing RFP labeling of β-cells.

Proliferation assay EdU obtained from the Click-iT® EdU Alexa Fluor ® 647 Imaging Kit from Invitrogen™ was utilized. At 4 dpf following MTZ treatment and sorting of fully-ablated embryos, EdU in DMSO (7 μM) was added to the 24-well plates containing the embryos along with the compounds. Following a 48 hour regeneration period at 35°C, EdU was incorporated into the regenerated cells. Immunostaining was performed to visualize the number of cells that incorporated EdU under the Zeiss LSM 700-405 confocal microscope.

Fixation of embryos At 6 dpf, following the regeneration period, select embryos were saved for staining and confocal imaging to obtain accurate cell counts for statistical analysis, as well as high-resolution images. Approximately 10 embryos were placed into a 2 mL microcentrifuge tube and all water was removed from the tube. PEM fixation buffer (0.1 M PIPES pH 6.95, 2 mM EGTA, 1 mM $MgSO_4$, 945 μL) and 2% formaldehyde in PEM (55 μL) were added to the tube. The embryos were able stored at -20°C for up to 2 weeks for confocal visualization. After this time period, fluorescence proteins begin to degrade and therefore lose brightness and visibility.

CHAPTER 4

RESULTS AND DISCUSSION

Preliminary chemical screening results Chemical screening of the Selleck stem cell library consistently yielded one compound as "very hopeful" for β-cell regenerative properties. The small molecule BX-795 (**Figure 7**) showed much larger β-cell mass upon treatment with 25 μM of the compound, compared to the negative control. This compound was retested ten times throughout the course of the screening by viewing β-cell mass size under the GFP filter (**Figure 8**). Approximately 80% of the embryos treated with BX-795 were found to have a β-cell mass size that appeared to be between the size of the negative control and the positive control following 48 hours of treatment. BX-795 is associated with anti-inflammation, consistent with findings in a similar study by Andersson et al[30]. It functions by inhibiting the activation of TANK-binding kinase 1 (TBK-1) and Inhibitor of nuclear factor kappa-B kinase subunit epsilon (IKKε), which inhibits the Toll-like receptor signaling pathway and therefore inhibits activation of the NFκB signaling pathway. These results are consistent with and appear to provide further insight previous findings that show that inhibitors of these protein kinases improve obesity-related metabolic dysfunctions, particularly insulin resistance and tissue inflammation[31]. This compound was hence investigated further to determine the extent of its β-cell regenerative capacities.

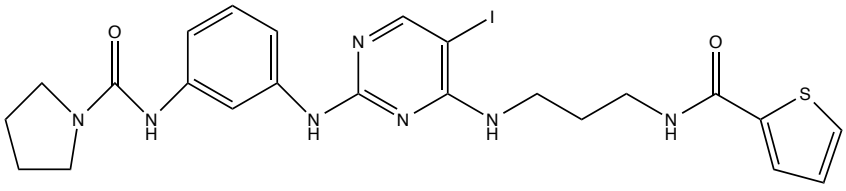
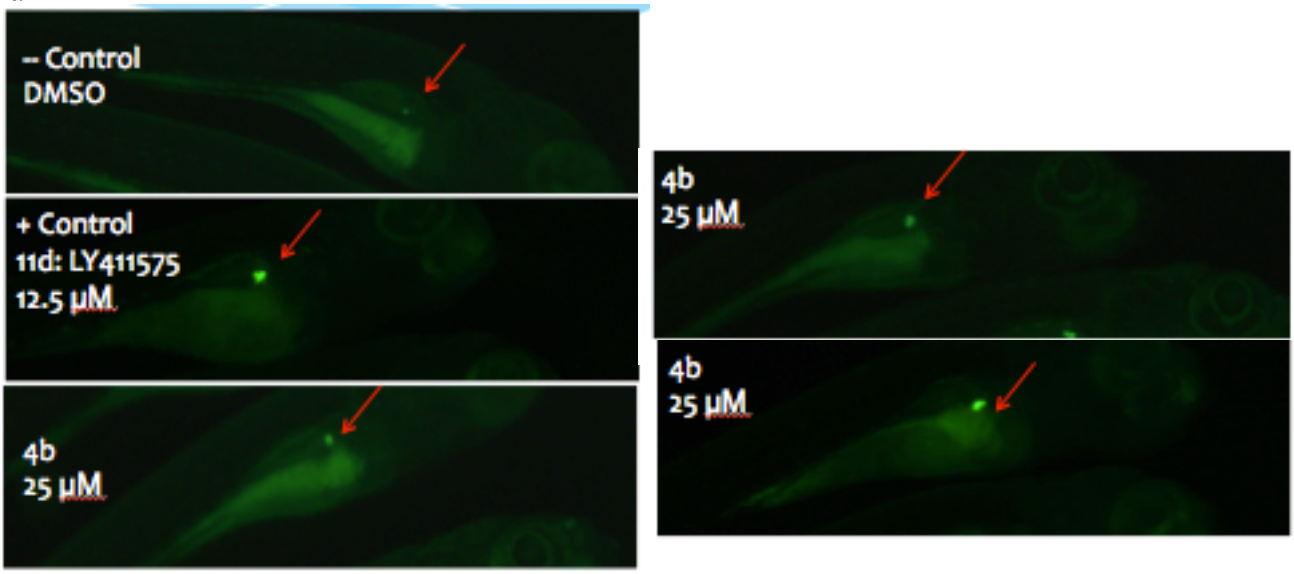


Figure 7: Chemical structure BX-795. The compound has a chemical formula of $C_{23}H_{26}IN_7O_2S$ and it known to be an inhibitor of TBK-1 and IKKε.

a



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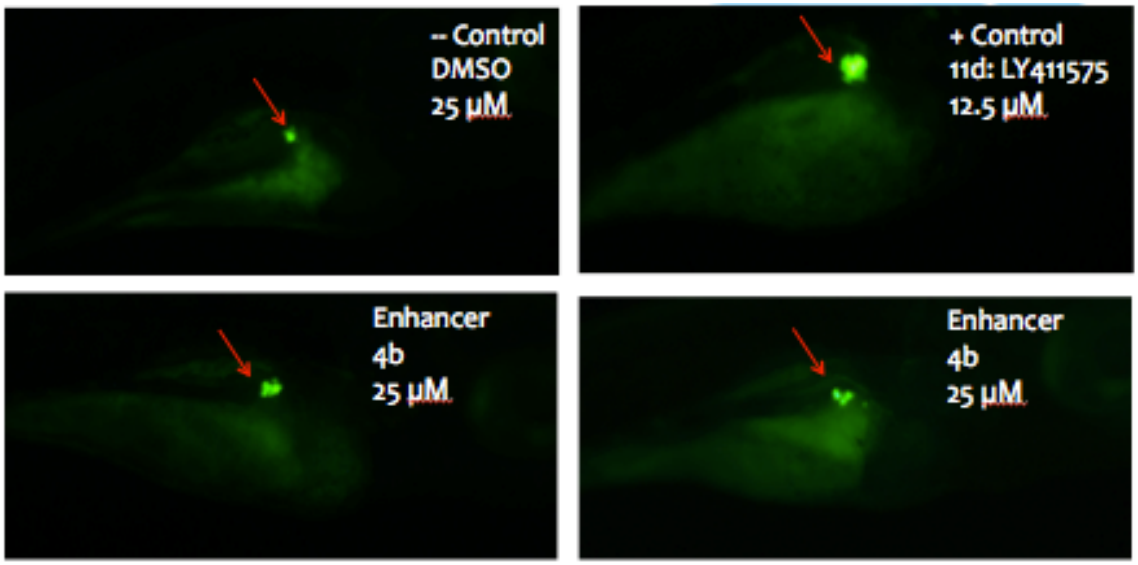

Figure 8: Preliminary results. a) First trial of embryos treated with DMSO, positive control compound, and compound 4b (BX-795) at 6 dpf under green fluorescent protein filter. A bright dot representing regeneration of β-cell mass is present in each of these embryos. The three trials for BX-795 shown are representative of repeated results showing a visibly larger regenerated β-cell mass for embryos treated with BX-795, compared to the negative control DMSO. The sizes of these cell masses are smaller than those of embryos treated with the positive control compound LY411575, but are closer to the size of this positive control than that of the negative control. b) Second trial of embryos treated with DMSO, positive control compound, and compound 4b (BX-795) at 6 dpf under green fluorescent protein filter. A bright dot representing regeneration of β-cell mass is present in each of these embryos. The results are consistent with those of the first trial, with BX-795 showing more β-cell regeneration than the negative control.

BX-795 increases regeneration rather than survival of β-cells To determine whether the results seen in the preliminary study are caused by BX-795 promoting of regeneration of new β-cells rather than survival of β-cells following MTZ treatment, the fate of β-cells was tracked by labeling. The GFP characteristic of the kaede protein in *Tg(ins:Kaede)* embyros was photoconverted from green to red by exposing the β-cells to UV light at 3 dpf, prior to treating the embryos with MTZ. The embryos were treated for the next 48 hours with MTZ to ablate the β-cells, and following ablation the embryos were treated with BX-795. β-cells that survived the ablation appeared yellow, due to overlap of expression of both green and red kaede protein, while the cells β-cells that were newly regenerated only appeared green. It was found that the size of the green β-cell mass was consistently larger for compounds exposed to BX-795 compared to those exposed to the control. When yellow cells were present, the size of this cell mass was consistently small for all treatment groups (**Figures 9-10**). Additionally, the size of the yellow cell mass did not appear increased for the BX-795 treatment group. Based upon these findings, BX-795 causes an increase in regeneration rather than survival of β-cells.

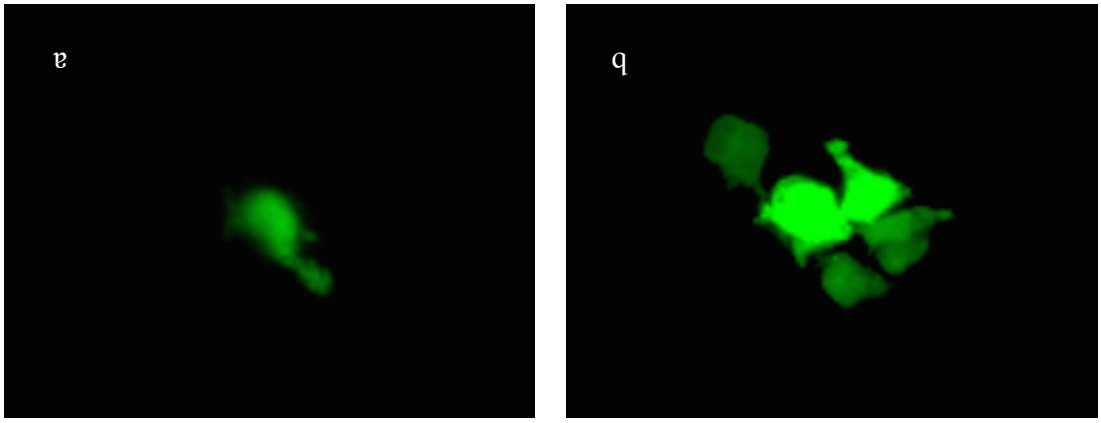

Figure 9: BX-795 promotes increase in number of β-cells following ablation. These images, obtained using the Zeiss LSM 700-405 confocal microscope, show the effect of BX-795 on β-cell regeneration following MTZ-mediated ablation from 3 dpf to 4 dpf and chemical treatment from 4 dpf to 6 dpf. a) Regenerated β-cell mass of control embryo expressing *Tg(ins:Kaede*) at 6 dpf. Only a small number of cells were observed to have regenerated. b) Regenerated β-cell mass of embryo expressing *Tg(ins:Kaede*) treated with 25 μM BX-795 from 4 dpf to 6 dpf at 6 dpf. A visibly larger number of regenerated β-cells were observed for this treatment group compared to the control group.

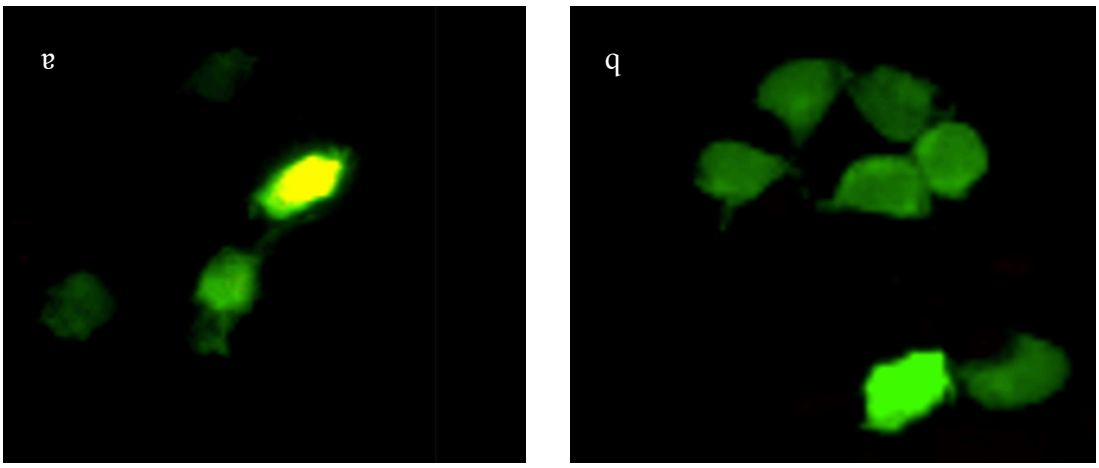

Figure 10: BX-795 promotes regeneration rather than survival of β-cells. At 3 dpf, the *Tg(ins:Kaede)*-expressing β-cells were photoconverted from green to red by exposing them to UV light and then treated with MTZ for 24 hours. After two days of regeneration, through 6 dpf, β-cells that survived the MTZ ablation appeared yellow, due to overlap of red and green cells, while those that truly regenerated were green only. a) Confocal images of the β-cells of a control embryo, which was treated with only DMSO, at 6 dpf. Only one β-cell survived ablation in this embryo. b) Confocal images of β-cells at 6 dpf of an embryo treated with BX-795. No β-cells were shown to have survived ablation in this particular embryo. Consistent with Figure 9, the size of the β-cell mass is larger and the number of β-cells is greater for the BX-795 treated embryos than the control embryos.

BX-795 does not impact β-cell regeneration during normal development The effect of BX-795 was tested on development of the pancreas without MTZ-induced ablation of the β-cells. This was performed to determine whether BX-795 causes a general increase in number of β-cells even without any stress having acted upon the cells or has therapeutic action that only functions in response to stresses, such as ablation. The general chemical screening protocol was followed, with compound treatment from 4-6 dpf but without ablation from 3-4 dpf. It was found that BX-795 did not cause a significant and visible increase in the size of the β-cell mass (**Figure 11**). This gives initial evidence that BX-795 may function by restoring an optimal number of β-cells, rather than causing unnecessary increase in the number of β-cells beyond that which is necessary to maintain proper blood glucose levels. Based upon this hypothesis, BX-795 will not increase β-

cell mass to levels that can cause higher-than-normal insulin levels and therefore lower-than-normal blood glucose levels.

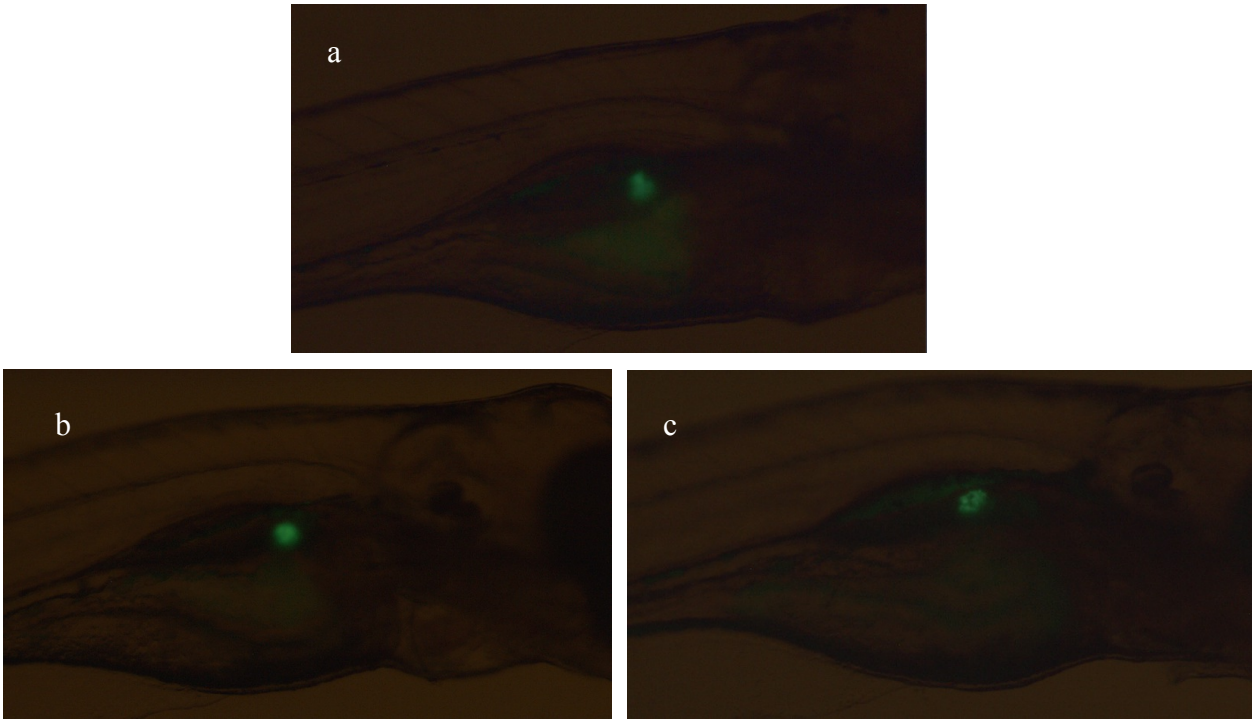

Figure 11: BX-795 exposure does not have a significant effect on size of β-cell mass during normal development, without ablation by MTZ. a) A control embryo, which was treated with DMSO only; b) First trial of treatment of embryos treated with BX-795; c) Second trial of embryos treated with BX-795. If the β-cells are not ablated prior to compound treatment, there is no significant difference in the size of the β-cell mass of the embryos treated with the control and the embryos treated with BX-795.

Predicted molecular significance of BX-795 The compound BX-795 is known to act as an anti-inflammatory agent and inhibits the catalytic activity of the protein kinases tank-binding kinase 1 (TBK1) and Inhibitor of nuclear factor kappa-B kinase subunit epsilon (IKKε) of the toll-like receptor signaling pathway, has been found to have a constructive effect on the regeneration of β-cells in the zebrafish model organism. Amlexanox, another TBK1 and IKKε inhibitor and a drug used to treat asthma that is a TBK1 and IKKε inhibitor, has been found to alleviate obesity and obesity-induced health problems in mice. This drug decreased insulin

resistance as well as expression of inflammatory markers.[30] This study deduced that increased activity of TBK1 and IKKε by ATP-phosphorylation is the precursor to increased activity of the NFκB signaling pathway in the adipose tissue and liver of obese mammals. The findings of our study thus far provide further evidence for these results by exhibiting that production of insulin may be increased by inhibition of TBK1 and IKKε. All in all, the results of this study obtained thusfar call for a paradigm shift toward a new method of treatment for Type I diabetes that involves directly targeting the β-cells to proliferate and therefore produce more insulin. Nonetheless, a significant amount of further testing is necessary to confirm these results and to propose the mechanism of action of BX-795 in the context of insulin regeneration.

CHAPTER 5

FUTURE WORK

CURRENT STUDIES

The following studies are currently in progress. Results will be obtained during Summer 2015.

Proliferation assay with BX-795 Results from a proliferation assay is a means of verifying the increase in proliferation of β-cells caused by treatment with BX-795. This is a method of assessing whether or not selected compounds caused increase in rates of mitosis, by way of measuring the incorporation of 5-ethynyl-2'-deoxyuridine (EdU) into the β-cells. EdU is an nucleotide analog of thymine, and its incorporation into β-cells is indication of DNA replication characteristic of the S phase of interphase that leads to mitosis. The incorporation can be visualized and quantified by staining of the β-cells and counting the number of β-cells that show a particular type of staining that indicates presence of EdU. A higher number of cells incorporating EdU in the embryos treated with BX-795 compared to those treated with the control would verify BX-795 has the effect of inducing mitosis and therefore increasing cell proliferation.

Testing of compounds with analogous targets to those of BX-795 The compound BX-795 is known to act as an anti-inflammatory agent and inhibits the catalytic activity of the protein kinases TBK1 and IKKε of the toll-like receptor signaling pathway. Three compounds that are also inhibitors of TBK1 and IKKε, namely Amlexanox, CYT387, and MRT67307, are being tested at similar working concentrations to those of BX-795 to determine if they have the same regenerative effect on BX-795. This experiment is intended to confirm the targets on which the remainder of the study will focus.

FUTURE STUDIES

The following experiments will be completed by my graduate mentor Jin Xu during Summer 2015.

Confocal imaging and statistical analysis Visualizing the number β-cells following regeneration using confocal microscopy (and in some situations nuclei staining) will allow for accurate counting of number of β-cells regenerated. This will allow for determination of statistical significance of the number of β-cells regenerated as a result of treatment with BX0795 versus treatment with the control compound.

Effects of BX-795 on other cell types The effect of BX-795 on proliferation of cell types other than β-cells, including endocrine α-cells (responsible for controlling glucagon levels) and endocrine δ-cells (responsible for controlling somatostatin levels), as well as liver and gut cells, will be tested. The significance of these experiments is to determine whether BX-795 specifically induces proliferation of β-cells or causes a general increase in proliferation of endocrine and/or other types of cells. This will be tested using the chemical screening protocol above but with transgenic lines that express NTR in these specific cell types to allow for targeted ablation of these cell types. The proliferation assay will also be performed and EdU incorporation will be quantified to determine the effect of BX-795.

Effects of BX-795 on β-cells in the mouse model The effect of BX-795 on β-cell proliferation and regeneration in mice will be tested to determine if analogous results to those observed in zebrafish are observed in mice. Trangenic mice will be injected with streptozotocin (STZ) to deplete their β-cells and the effectiveness of BX-795 in promoting regeneration of β-cells will be observed. This study will aim to prove that the effect of BX-795 on zebrafish is also

conserved in mammals, which will further the potential for utilizing compounds with analogous targets for formulation of drugs.

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VITA

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PITHADIA was born in Des Plaines, Illinois. She grew up in Columbus, Georgia and received a B.S. in Biochemistry from the Georgia Institute of Technology in 2015. She will begin medical school in Fall 2015. When she is not working on her research, Miss Pithadia enjoys Indian dance and cooking.